Comparative study between biochemical and histological methods and image analysis in liver iron overload

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SUMMARY Iron overload has been measured in 100 hepatic biopsies by three different methods: (i) biochemical assay of the liver iron concentration (LIC), (ii) histological grading (HISTO) and (iii) automated image analysis with a Leitz Texture Analysis System by estimating two parameters, (a) the total iron area (TIA) and (b) the first grey level step (FGLS) at which the iron is detected. Image analysis appears as a specific, sensitive, quick, reproducible and valid method.

The direct measurement of hepatic iron overload is usually based upon two complementary methods: biochemical determination of liver iron concentration (LIC) and histological examination (HISTO). The biochemical assay is considered as the reference method.[1][2] The histological reading provides a more approximate measurement than biochemistry, although it is generally satisfactory[2] and provides the necessary qualitative information concerning cellular distribution of iron and any associated lesions. Thus it is necessary to use both methods but they are time- and staff-consuming. The exact measurement of the liver iron overload is of prognostic and therapeutic value. Consequently it has been important to us to determine whether automated image analysis permits a quantitative and qualitative estimation of hepatic iron overload sufficiently reliably and quickly to replace the standard methods. The aim of the study presented here is to evaluate the specificity, the validity and the rapidity of the measurement of hepatic iron by automated image analysis.

Material and methods

One hundred liver biopsies transparietally, transjugularly or surgically performed from 100 patients have been studied (Table 1). The iron was measured on each biopsy using three different methods:
(i) biochemical determination of liver iron con-

Table 1 *Patients diagnosis and liver fibrosis quantification*

Diagnosis	No of patients	Fibrosis				
		0	+	++	+++	++++
Idiopathic haemo-chromatosis	39	6	5	8	12	8
Alcoholic liver diseases	35	6	11	7	4	7
Non-alcoholic liver diseases	26	16	4	4	1	1
Total	100	28	20	19	17	16

Histological grading: 0 = no fibrosis
+ = mild portal fibrosis
++ = portal and periportal fibrosis
+++ = dissecting fibrosis
++++ = cirrhosis

centration according to the method of Barry and Sherlock[4] (LIC; N ≤ 150 μg/100 mg dry weight).
(ii) histological examination (HISTO) of 2 μm thin sections stained according to the method of Tirmann and Schmelzer[4] and counter-stained with nuclear red. An original classification has been used which consists of five classes taking into account not only parenchymal iron but also iron in Kupffer cells and in fibrosis (Table 2). The elementary score (score A) represents for parenchymal cells the percentage of cells presenting iron; score B is obtained by multiplying the score A for hepatocytes by a coefficient of 3. The total, scored on a scale of 0-20, is then divided into five classes (0, I, II, III, IV).
(iii) automated image analysis with a Leitz Texture Analysis System (TAS). This device is composed of a

Table 2 *Histological grading*

Iron	Hepatocytes (H)		Kupffer cells (K)		Fibrosis (F)
Score A	0-4		0-4		0-4
Coefficient	3		1		1
Score B	0-12		0-4		0-4
Total score	0	1-5	6-10	11-15	16-20
(H + K + F)	↓	↓	↓	↓	↓
Final grade	0	I	II	III	IV

microscope, a TV camera and a hardwired system allowing one to process the image. The detection is performed by grey-level (GL) thresholding of the video signal. One hundred different grey-levels can be detected, from black (GL 0) to white (GL 100). Thus, it is possible to select the grey-level(s) corresponding to the structures under study. The binary image is visualised on a TV monitor. It can then be interactively processed, and measurements of various parameters can be asked for. With an adequate program the different steps of detection and measurements, as well as scanning stage movements, are automatically executed. The output of the results and statistics is displayed by a printer.

The morphometric study was done on serial 2μm thin sections situated immediately adjacent to those used for histological reading. The preparations were stained according to the method of Tirmann and Schmelzer[4] without any counter-staining. A differential densitometric analysis scanning of the grey-level scale of 20 five grey-level steps was done with a × 25 objective on five different zones of 5-10-15 or 20 fields per slide without image transformation. Two parameters were evaluated: the total iron area (TIA) representing the mean value of the iron areas in the five zones expressed as a percentage of the tissue area under study and the first grey-level step (FGLS) at which 0·5% of the detected iron, or more, appears. The grey-level steps are numbered from 0 (black) to 20 (white) (Fig. 1). Thus a low FGLS corresponds to a heavy overload and a high FGLS corresponds to a slight overcharge.

The statistical tests used were the non-parametric test of Mann and Whitney, and the calculation of the linear regression coefficient. The standard deviation was calculated from the mean value.

Results

MEASUREMENT OF THE IRON OVERLOAD BY THE THREE METHODS

Liver iron concentration
The LIC values ranged from 20 to 2 450 μg/100 mg dry weight. Thirty-three patients had a normal LIC ($\leqslant$ 150). Iron overload was slight in 20 cases (150-250), moderate in 16 (250-500), important in 11 (500-1100) and severe in 20 ($\geqslant$ 1100).

Histological reading
The biopsies were distributed as follows: 21 in class 0, 25 in class I, 20 in class II, 10 in class III and 24 in class IV.
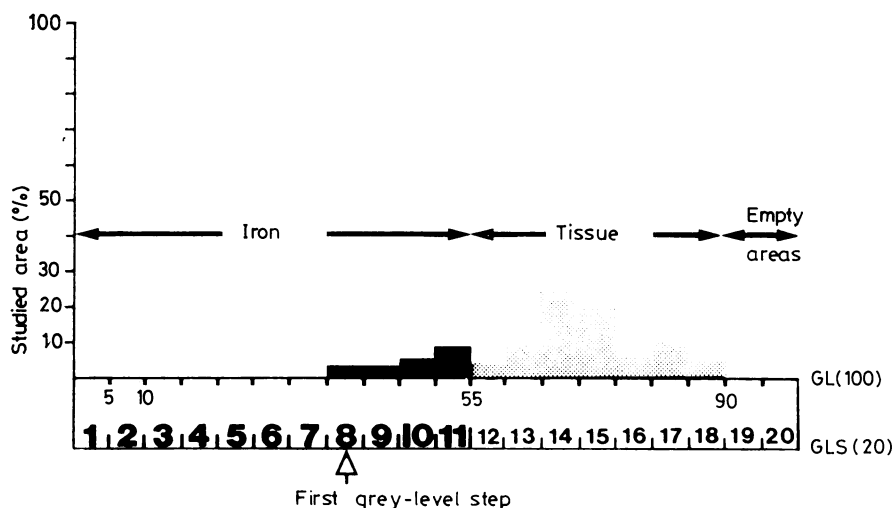

Fig. 1 *Example of an iron overloaded preparation: iron area occupied at each grey-level step (GLS; I to II) v total studied area. The total iron area (TIA) appears black. FGLS represents the first grey-level step at which iron appears (here, FGLS 8).*

Image analysis
The total iron area (TIA) values ranged from 0·005 to 54·5%; the iron appeared from FGLS 2 (severe overload) to FGLS 11 (slight or absent overload).

CORRELATIONS

Classical methods
As has been previously reported[2] the LIC-HISTO correlation (Fig. 2) is generally satisfactory. Histological classes 0 (LIC = 89·5 ± 16), I (LIC = 142 ± 13) and II (LIC = 296 ± 36) on the one hand, and

the agreement between the two measurements is not as good (r = 0·68) for high overload (LIC > 1000) as for moderate, slight or absent ones. The agreement between LIC and FGLS is also satisfactory (Fig. 4), FGLS allowing in contrast to TIA, the separation of high overloads in three groups: FGLS 2, FGLS 3 and FGLS 4 whose LIC values are respectively 1822 ± 105, 1454 ± 175 and 937 ± 129 (p < 10^{-3}).

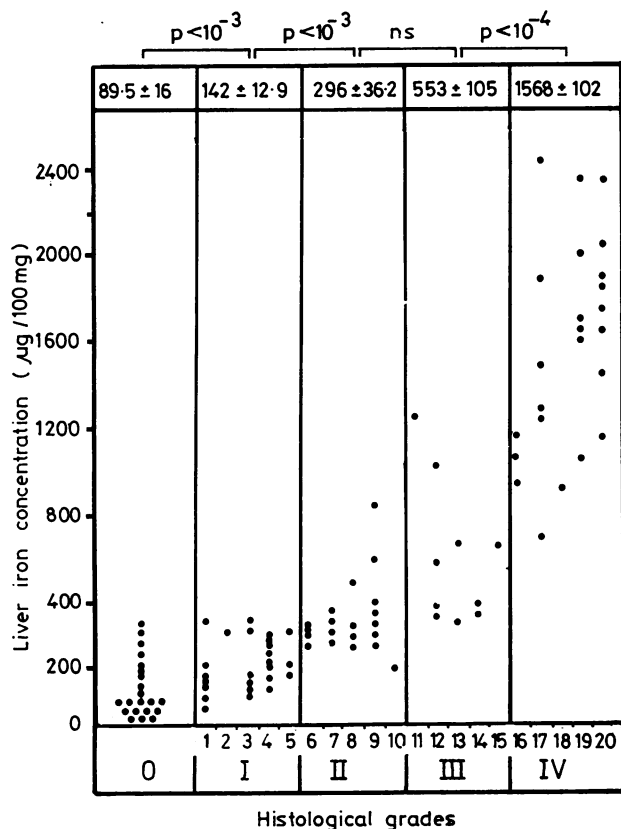*Histological reading-image analysis*
The HISTO-TIA correlation (Fig. 5) is not as good

Fig. 2 *Liver iron concentration (LIC; μg/100 mg; mean standard deviation) v histological grades (0 to 20) and classes (0 to IV). Significance is appreciated according to the test of Mann and Whitney.*

classes III (LIC = 553 ± 105) and IV (LIC = 1568 ± 102) on the other hand have LIC values very different from one another (p < 10^{-3}). The only classes that clearly overlap are classes II and III.

Biochemical assay-image analysis
The LIC-TIA correlation (Fig. 3) is very good (r = 0·90). However, it should be pointed out that

as the preceding ones: histological classes I (TIA = 0·4 ± 0·08) and II (TIA = 2·5 ± 0·6) on the one hand and III (TIA = 9 ± 2·2) and IV (TIA = 31·8 ± 2·7) on the other are easily distinguished from one another by their TIA values (p < 10^{-2}), but a clear overlapping between classes 0 (TIA = 0·13 ± 0·04)—I and II—III can be seen. The HISTO-FGLS correlation (Fig. 6) is not at all satisfactory.
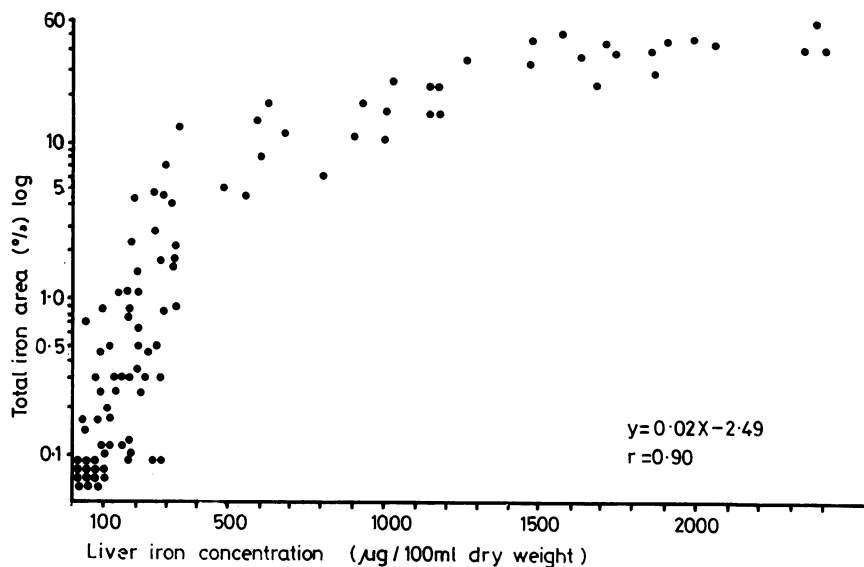
Fig. 3 *Total iron area (TIA; % of the total area under study; logarithmic co-ordinates v liver iron concentration (LIC; μg/100 mg). Significance is appreciated by calculation of the linear regression coefficient (r = 0·90; y = 0·02x − 2·49).*
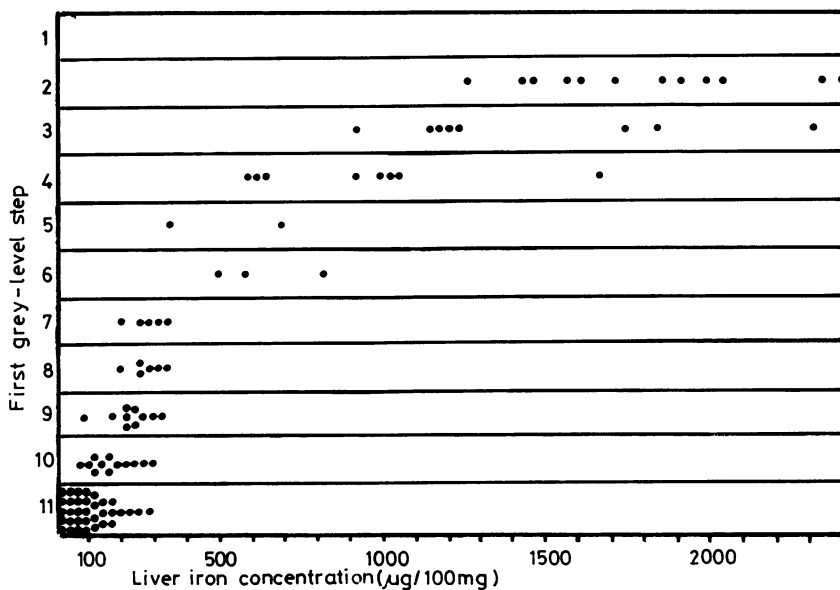

Fig. 4 *First grey-level step (FGLS; 1 to 11) v liver iron concentration (LIC; μg/100 mg).*
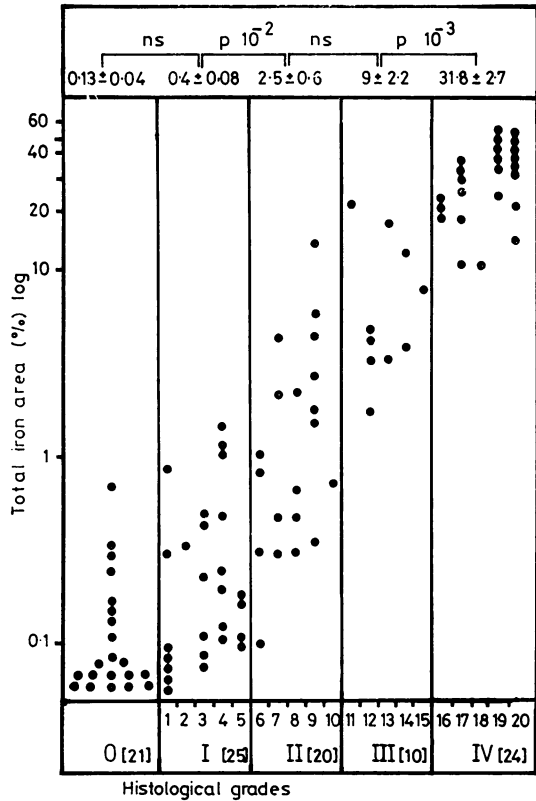
Fig. 5   *Total iron area (TIA; % of the total area under study; logarithmic co-ordinates; mean standard deviation) v histological grades (0 to 20) and classes (0 to IV).*
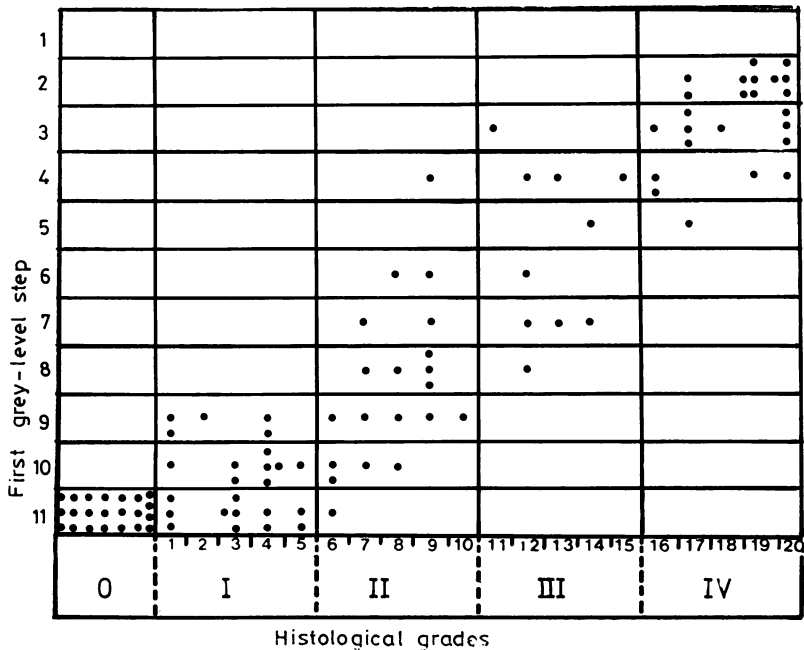
Fig. 6   *First grey-level step (FGLS; 1 to 11) v histological grades (0 to 20) and classes (0 to IV).*

Discussion

SPECIFICITY AND SENSITIVITY OF THE DETECTION BY IMAGE ANALYSIS

The detection of the iron stained according to the method of Tirmann and Schmelzer[4] is specific and sensitive as long as the preparations are not counterstained with nuclear red. The iron is detected at grey-levels ranging from 0 to 55, tissue structures and empty spaces being detected beyond the latter value (Fig. 1). However, false-positive determinations occur as the TIA values for the 21 biopsies classified in class 0 by the histologist (Fig. 5) are never null, although they are very low (0·004 to 0·7; 0·13 ± 0·04). The existence of these false-positive values can be explained by:

(i) the unavoidable "background noise" due to the presence of dusts. The value of this noise, as estimated on unstained or blank slides, is 0·05 to 0·1%.

(ii) the existence of artefacts (formol pigment, folding of the preparation dye spots) or scarce ferruginous granulations (or all of these) not taken into account by the histologist.

The automatic elimination of all the artefacts by the analyser was not done because of the high degree of shape-, size-, and staining- heterogeneity of the iron particles. However, it is sure that after elimination of gross artefact by visual control, only the iron is detected between grey-levels 0 and 55.

A few false-negative values also occur since at FGLS II one can find all the biopsies classified in class 0, but also 12 biopsies of histological class I (Fig. 6). This can be due to a slightly insufficient thresholding and/or an incomplete scanning of the preparation, although the quality of the correlations between image analysis, histology and biochemistry does not depend upon the number of analysed fields.

RELIABILITY OF THE MEASURED PARAMETERS

Reproducibility
In 30 cases randomly chosen, the measurements have been performed twice; there is a very good agreement for both TIA (r = 0·96) and FGLS (FGLS unchanged in 27 cases and varying by one unit in two cases of slight overload and one case of severe haemochromatosis). The automation of focusing and light adjustment should improve this good reproducibility.

Validity
The very good TIA-LIC correlation and the good FGLS-LIC and TIA-HISTO agreements allow us to state that image analysis is an alternative method for the quantification for hepatic iron overload. Three points must be emphasised: (i) the TIA allows a good discrimination between slight and moderate iron overcharges (histological classes 0-III; LIC < 1100) but is not more precise than histological reading in the quantification of severe overloads (LIC ⩾ 1100) independently of the fibrosis degree, (ii) on the contrary, the FGLS seems more discriminating for the high overloads than for the low ones and (iii) the HISTO-FGLS correlation is bad.

The TIA and the histological classification are relevant to two-dimensions and account only for areas but FGLS introduces a third dimension by an appreciation of the iron overload colorimetric density. This explains the disagreements between FGLS and HISTO, and the impossibility of distinguishing the high overloads from one another beyond a certain value of LIC (1100) by measuring TIA only. It is important to determine both TIA and FGLS in order to avoid the underestimation of the low iron overloads and to refine the measurements of high ones. Moreover an appreciation of cellular iron overload density should be related to class histological criteria.

Rapidity of the measurements
It takes the image analyser four seconds to process one field and two to six minutes per slide to perform the measurements and calculations in order to obtain the TIA and FGLS values.

The time-saving factor may be appreciable both for the pathologist and the biochemist. The manipulations can be done by a technician and in most cases the TAS estimation is a sufficient procedure for hepatic iron measurement.

Conclusion

Image analysis is a specific, sensitive, quick, reproducible and valid method for quantifying hepatic iron overload with good correlations between image analysis, chemistry and histology as long as the determination of the first grey-level step at which the iron appears is related to the calculation of the area occupied by the iron.

The reliability of the results allows the extension of its field of application to the measurement of new parameters such as cellular and lobular distribution of iron, size of the iron particles, especially in experimental iron overload.

References

[1] Barry M. Liver iron concentration, stainable iron and total body storage iron. *Gut* 1974;**15**:411-5.

2 Brissot P, Bourel M, Herry D, *et al.* Assessment of liver iron content in 271 patients: a re-evaluation of direct and indirect methods. *Gastroenterology* 1981;**80**:557-65.

3 Barry M, Sherlock S. Measurement of liver iron concentration in needle biopsy specimens. *Lancet* 1971;i: 100-3.

4 Lillie RD. *Histopathologic technic*. Philadelphia: Blakiston Company, 1948:123-4.

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